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Enhanced Diosgenin Production in Costus Speciosus Via inVitro Cultivation

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Abstract:
The present invention relates to an improved method for enhancing diosgenin production in *Costus speciosus* through in vitro cultivation. Diosgenin, a valuable precursor for steroidal drug synthesis, is traditionally obtained from field-grown plants, which are subject to environmental variations and long growth cycles. This invention provides a novel approach using optimized in vitro culture techniques, including Shoot induction, Shoot multiplication cultures, and Growth harmones added in culture to significantly enhance diosgenin biosynthesis. The method ensures higher yield, faster production, and controlled conditions, making it a sustainable and commercially viable alternative to conventional in vivo cultivation. Experimental results confirm a substantial increase in diosgenin content compared to naturally grown *Costus speciosus*.

1. Introduction

The present invention provides an optimized method for enhancing diosgenin production in *Costus speciosus* through in vitro cultivation. Diosgenin, a steroidal sapogenin, is widely used in the pharmaceutical industry as a precursor for corticosteroids and hormonal drugs. Traditional field-based cultivation of *Costus* speci osus poses challenges such as long growth cycles, seasonal variations, and inconsistent diosgenin yield. This invention overcomes these limitations by utilizing controlled in vitro culture techniques, leading to higher diosgenin production within a shorter time frame. In vitro cultivation has emerged as a promising alternative for the large-scale production of bioactive compounds, offering controlled conditions, rapid biomass accumulation, and enhanced metabolite synthesis. Tissue culture techniques such as shoot induction, shoot multiplication,root induction and the application of growth harmones have demonstrated their potential in increasing secondary metabolite production, including diosgenin.

The present invention addresses these limitations by providing an optimized in vitro culture method that significantly enhances diosgenin yield. By utilizing advanced culture strategies and biochemical elicitation, this approach ensures a sustainable and commercially viable alternative to conventional field-based cultivation. The optimized method not only improves diosgenin content but also enables year-round production, ensuring consistency in pharmaceutical applications. Diosgenin, a bioactive steroidal saponin, is a crucial precursor for the synthesis of corticosteroids, oral contraceptives, and other pharmaceutical steroids. It is primarily sourced from plants, with *Costus speciosus* being one of the potential natural reservoirs. Traditionally, diosgenin extraction is dependent on in vivo cultivation, which presents several challenges, including long growth cycles, seasonal dependency, and variability in yield due to environmental factors.

2. Material and Methods

Collection of plant material

In vitro micro propagation starts from selection of explants. The first step in this process is selection and collection of plants. Costus speciosus (J.Koen) certified plant were collected from Vindhya herbal Bhopal . Vindhya herbal known as a unit of M.P. State minor forest production center, cooperative federation limited .

Preparation of stock solution

The composition of MS (1962) is given in table -2.1

Preparation of stock solution of nutrients Macronutrients is given in table -2.2 Micronutrients in table -2.3, Stock solution for $Feso_4.7H_2O$, Na_2 EDTA H_2O is given in table-2.4, Myoinositol is given in table-2.5, Vitamins table -2.6 and stock solution for phytohormones are given in table -2.7 .

Sterilized double distilled water was used when we prepare culture media. pH of the media was adjusted by adding 1 N HCL or 1 N NaOH. Stock solutions individually or in groups are prepared well in advance and used sub-sequentially to prepare before experimentation. Stock solution after preparation are stored in refrigerator. Storage life of different stock solution varies.

Before using the stock solution; they were checked for any contamination & precipitation. The composition of MS basal media was used for all in vitro studies.

Macronutrients : For MS medium the stock solution was prepared Individually all the salts were separately dissolved in double distilled water. The solution was then poured to 1000 ml capacity volumetric flask and added double distilled water to make up the final volume .

Micronutrients: Accurately all the salts of micronutrients dissolved in 100 ml double distilled water. Final volume 500 ml. made up with distilled water and stored in a cool temperature.

Iron EDTA solution Stock solution of Iron EDTA was prepared at concentration of 100X. The salts were dissolved separately in distilled water. final volume 500ml by added distilled water. In a colored bottle in a refrigerator Iron stock was stored.

Myo-Inositol Stock solution of myo –inositol was separately as 50 X .

Carbon source Sucrose (2-4%) was used as source of energy & carbon. The sucrose in the medium is rapidly converted into glucose and fructose.

Vitamins: Pyridoxine (B6), nicotinic acid (B3)and thiamine(B1) are the most frequently used vitamins in tissue culture media . They are added in medium before autoclaving. . Cell growth promoted by pyridoxine and nicotinic acid and thiamine is required for most cultures.

Amino acids For morphogenesis the amino acids are very essential in tissue cultures specially All L forms of amino acids are commonly used.

Organic supplements :Organic supplements are coconut milk . Coconut milk contain a number of essential nutrients and vitamins.

Solidifying agents : 8gm Agar is used as a solidifying agent.

Plant growth regulators :In recent work phytoharmones have been used individually and in combination with others. The concentrations are mentioned in tables. Since these are photosensitive chemicals so sotored in dark & cool place.The hormones used are Auxin ,Cytokinin ,Gibberellins .

Table 2.1 : Composition of MS Media used for in vitro culture

Constituents	Murashige and Skoog (MS 1962) mg/L
A. Inorganic	
Macronutrients	
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$NaH_2PO_4.H_2O$	-------
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$NH_4H_2PO_4$	-------
Micronutrients	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MOO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
B. Organic	
Thiamine HCL	0.1
Pyridoxine HCL	0.5
Nicotinic Acid	0.5
Myo-inositol	100
Glycine	2.0
Sucrose	30

TABLE- 2.2 SOLUTION OF MS (1962) MACRO SALTS (X 10)				
Constituents	**Amount (mg/l) Present in original medium**	**Amount (g/l) to be taken for stock solution (X 100)**	**Final Vol. Of S.Solu. (ml)**	**Amount to be used / litre (ml)**
NH_4NO_3	1650	16.5		
KNO_3	1900	19.0		
$CaCl_2.H_2O$	440	4.4	1000	100
$MgSo_4 7H_2O$	370	3.7		
KH_2PO_4	170	1.7		

TABLE-2.3 SOLUTION OF MS (1962) MICRO SALTS (X 100)				
Constituents	**Amount (mg/l) present in orginal medium**	**Amount (gm /l) to be taken for stock solu. (x100)**	**Final vol. Of S.Solu. (ml)**	**Amount to be used liter(ml)**
Kl	0.83	83		
H3Bo3	6.2	620		
MnSo4, 4H20	22.3	2230		
ZnSO4, 7H2O	8.6	860	500	5
Na2MoO4,2HO	0.25	25		
CuSo4,5H2O	0.025	2.5		
CoCl2, 6H2O	0.025	2.5		

TABLE-2.4 STOCK SOLUTION OF MS (1962). IRON SOURCE (X 100)				
Constituents	**Amount (mg/l) present in orginal medium**	**Amount (gm/l) to be taken for stock solution (x100)**	**Final vol. Of S.Solu. (Ml)**	**Amount to be used/1/ml**
$FeSO_4$, $7H_20$	27.8	2.78		
$Na_2EDTA,2H_2O$	37.3	3.73	500	5

TABLE-2.5 STOCK SOLUTION OF MS (1962) INOSITOL (X 50)				
Constituents	**Amount (mg/l) present in orginal medium**	**Amount (gm/l) to be taken for stock solu. (x100)**	**Final vol. Of S.Solu. (Ml)**	**Amount to be used/1/ml**
Myo-inositol	100	5	250	5

TABLE-2.6 STOCK SOLUTION OF MS 1962 VITAMINS (X 100)				
Constituents	**Amount (mg/1) present in orginal medium**	**Amount (mg/1) to be taken for stock solu. (x100)**	**Final vol. Of S.Solu. (ml)**	**Amount to be used/1/ml**
Thiamine HCL	0.1	10		
Nicotinic Acid	0.5	50	500	5

Pyrodoxine HCL	0.5	50		
Glycine	2.0	200		

TABLE :2.7 STOCK SOLUTION OF PLANT GROWTH HORMONES

Phytoharmones	Mol.wt	Required amt. of Stock solu.	Amt.of solvent required	Amt.of water to be added	Final volume of stock solution	Final Conc .(mg/ l)
Auxin						
IAA	175.18	10	**1ml(0.1)NaOH**	99	100	0.1
NAA	186.20	10	1ml(0.1)NaOH	99	100	0.1
IBA	203.23	10	1ml(0.1)NaOH	99	100	0.1
Cytokinin						
BAP	225.20	10	1ml(0.1)HCL	99	100	0.1
KN	215.21	10	1ml(0.1) HCL	99	100	0.1

Culture media Preparation

The culture media prepared by using sterilized double distilled water . By adding 1N HCL or 1N NaOH the pH was adjusted. By autoclaving at 121°C, 15 Psi for 20-25 minutes. Medium containing flasks and tubes were finally sterilized . At room temperature autoclaved medium were cooled and used for inoculation of different explants.

The preparation of one liter MS (1962) medium involves following steps. In 500 ml of DDW 30 grams (3% w/v) of sucrose is dissolved. 100 ml of macro salts, 5 ml of microsalts, 5ml of vitamins, 5ml myoinosital, and 5ml of iron stock solution are added to the above solution. Growth hormones were added as per experiment. The volume of medium is made up to 1000 ml by adding DDW. The pH is adjusted between 5.2-5.8

by adding 1N NaOH or 1N HCl 8g (0.8%) of agar is added and solution is heated above 40°C to dissolve agar . Media is poured in Petri plates, vials or culture vessels as per requirement. The culture vials are finally plugged. Labeled and autoclaved at 15 lbs 121^0 C for 15-20 minutes. The volume of the medium in the vessel determined sterilization process .Autoclaving for long time may cause braking and denaturation of various media ingredients.

Inoculation of explants After sterilization of explants and media ,under aseptic condition sterile explants were transferred into media filled sterile bottles using sterile forceps. Inoculated cultures were kept in the culture room under aseptic and controlled conditions 16 hours photoperiod of 2000 lux and temperature 25^0C. On initiation of shoots the effect of season, age of explants and the effect of various growth hormones were studied .

Murashige and Skoog (1962) media were used for inoculation of rhizome. Culture medium with cytokinin (BAP)and auxin (NAA) (0.5 -2.0)mg/l alone and in combination . Sucrose 30 gm and agar 4 g/l were added .For the shoot induction in addition auxins (NAA 0.1-1.0 mg/l) was used .

Multiplication of shoots

The shoot starts to initiate after few days of inoculation. The initiated shoot were sub cultured in the multiplication medium.

This medium differs from the initiation medium in that it contains higher levels of cytokinins. Experiment were also conducted to check the effect of higher concentration of cytokinin alone and in combination with NAA. Number of experiment were carried out to studied the rapid multiplication of shoots.

At weekly interval explants initiation,regeneration were studied . As a parameter initiated shoot number and regenerated shoots hight were taken . The measurement was taken as % shoot response i.e developed multiple number of shoot and shoot length .

ROOT INITIATION The shoots inoculated into a rooting medium to obtain full plants, which is different from the shoot multiplication medium. After few times the multiplied regenerated shoots were separated and inoculated for root induction in the medium containing auxins like NAA, IBA and Activated charcoal (AC) the growth parameter was observed as percentage of root initiation, No. of roots formed. The roots formed in vitro are usually different morphologically than that of normal.

Table 1 Medium Used in Shoot induction of *Costus speciosus*

C1	1.0 BAP
C2	2.0 BAP
C3	3.0 BAP
C4	4.0 BAP
C5	1.0 BAP + 0.5 NAA
C6	2.0 BAP+0.5 NAA
C7	3.0 BAP+0.5 NAA+1.0 KN
C8	1.0 BAP+0.5 NAA+1.0KN
C9	4.0 BAP+0.5 NAA+1.0 KN
C10	4.0 BAP+0.5 NAA+10 AS
C11	4.0 BAP+0.5 NAA+ 20 AS

Table 2 -Different media used for the multiplication of shoot of the *Costus speciosus*.

Media code	MS+ Auxin/Cytokinin mg/l
C.M.1	**3.0**
C.M.2	**4.0**
C.M.3	**5.0**
C.M.4	**6.0**
C.M.5	**4.0 BAP+0.5 NAA**
C.M.6	**5.0 BAP+0.5 NAA**
C.M.7	**6.0 BAP+0.5 NAA 1.0 KN**
C.M.8	**5.0BAP+0.5 NAA +1.0 KN**
C.M.9	**5.0 BAP +0.5 NAA +20 AS**
C.M.10	**6.0 BAP +0.5 NAA +20 AS**

Table 3 Different media used for root production

Media code	Media MS +GR (mg/ l).
CR.1	10 AC
CR.2	**1.0 IBA.**
CR.3	**0.5 IBA.**
CR.4	**20 AC.**
CR.5	**2.0 IBA.**
CR.6	**0.5 NAA.**
CR.7	**1.0 NAA.**
CR.8	**MS +2.0 NAA.**
CR.9	**MS +0.5 IAA.**
CR.10	**MS +1.0 IAA.**

Phytochemical extraction and analysis:

Rhizome explant of Costus speciosus (J.Koen) were collected .After washing and cleaning rhizome cut into small pieces at room temperature shade dried.The powdered plant material were used for the extraction of secondary metabolites.

Section A

There are different methods of extraction, In the research work two types of extraction methods have been tried . (maceration and soxhlet extraction).

1.Maceration at room temperature (25° C) : 500 gm dried powdered material rhizome was macerated separately with methanol for 2 days with occasional stirring (Harborne 1998) . Using a rotator evaporator at 400 °C under reduced pressure the filtrates were evaporated to dryness .

2.Soxhlet extraction : 500 gms of dried plant material of *Costus speciosus* (J.Koen) was extracted in 95 % methanol in soxhlet apparatus for 6 hours.

Using a rotator evaporator at 400 °C under reduced pressure the extract was concentrated . The crude methanol extract was successively obtained . The percentage yields of extracts were calculated. Determination of percentage yields Calculation of percentage yield The percentage of the extract yield was calculated by using formula

$$\% \text{ Yield} = \frac{\text{Weight of extract}}{\text{Weight of powdered drug taken}} \times 100$$

Section B

Thin layer Chromatography (TLC) Methodology: Dried powdered material of Costus speciosus(J.Koen) was extracted with methanol as solvents using maceration process for 48 hours, filtered and dried using vacuum evaporator at 40°C.

The extract phytochemicals were prepared into a stock solution of 100 mg/ml concentration that was used for TLC analysis.

TLC Analysis for Qualitative Detection of Diosgenin & Colchicin (Saponin & alkaloid respectively)Chromatography conditions:

Table 3.1: Description of chromatographic conditions which were applied on methanolic extracts of C. speciosus.

S.N.	Particulars	Conditions	Make
1	TLC Plate (Stationary phase)	Thin layer silica gel 60G F_{254} DC Kieselgel 60 plates (5×7 cm)	Merk
2	Solvent System (Mobile phase) for Saponin	Toluene: Ethyl acetate: Acetic acid: Formic acid (ratio= 4:3:1:1)	Rankem and SD Fine
3	Solvent System (Mobile phase) for Alkaloid	Toluene: Ethyl acetate: Methanol: Ammonia(ratio= 30:20:20:0.1)	Rankem and SD Fine
4	Saponin Standard	1 µg per ml stock solution	HiMedia
5	Alkaloid standared	1 µg per ml stock solution	HiMedia
6	*Extract 1*	Methanolic extract	From natural plant
7	*Extract 2*	Methanolic extract	From tissue cultured plant

Table 3.2: Observation and results TLC profiling of standard markers on plate in chromatographic condition.

S.N.	Sample Used	No. of band/spot (At Short wavelength)	No. of band/spot (At Long wavelength)
1	*Diosgenin(std.)*	1	1
2	*Colchicin(std.)*	1	1

Calculation of R_f Value:

$$R_f \text{ (Retention factor)} = \frac{\text{Distance travelled by Spot}}{\text{Distance travelled by Solvent}}$$

Table 3.3 : TLC chromatogram details of methanolic rhizome extract of Naturally and tissue cultured *C. speciosus* methanolic extract in chromatography conditions compared to Diosgenin in both short & Long UV wavelength

Sample extract	*Extract 1 and Extract 2*
Mobile Phase	Toluene: Ethyl acetate: Acetic acid: Formic acid (ratio= 4:3:1:1)
Plate Tag	Plate 1
Spot description from left to right on plate	Diosgenin, extract 1 and extract 2 respectively
Distance travelled by mobile phase	7.0 cm
Number of spots at short & Long wavelength	6 & 7 Respectively
Visibility	Sharp
Distances of spots from start point of each spot (from bottom to top)	1.0 cm, 3.2 cm, 4.2 cm, 5.5 cm, 6.0 cm and 6.3 cm
Any Match with Diosgenin Standard	Yes
R_f Values of each spot (from bottom to top)	0.14, 0.45, 0.5, 0.6, 0.78, 0.85 and 0.9 respectively

***Extract 1 :Naturally grown**
Extract 2 :Tissue culture grown

Table 3.4 : TLC chromatogram details of methanolic rhizome extract of Naturally and Tissue cultured *C. speciosus* methanolic extract in chromatography condition compared to Colchicin in both short & Long UV wavelength .

Sample extract	*Extract 1 and Extract 2*
Mobile Phase	Toluene: Ethyl acetate: Methanol: Ammonia (ratio= 30:20:20:0.1)
Plate Tag	Plate 2
Spot description from left to right on plate	Colchicin, extract 1 and extract 2 respectively
Distance travelled by mobile phase	5 cm
Number of spots at short & long wavelength	5 & 6 Respectively
Visibility	Sharp
Distances of spots from start point of each spot (from bottom to top)	0.9 cm, 1.5 cm, 2.9 cm 3.8 cm, 5.0 cm and 5.6 cm
Any Match with Colchicin Standard	Yes
R_f Values of each spot (from bottom to top)	0.12, 0.21, 0.41, 0.54 0.71 and 0.8 respectively

***Extract 1 :Naturally grown**
Extract 2 :Tissue culture grown

Section C

3.11 (HPLC) analysis for Phyto-chemicals: HPLC is a technique in which components can be separated , quantify and identify from the mixture. For a accurate analysis of a particular compound there are some parameters which are perform as a standard .

The result may be greatly affected when parameter changed. Commonly used parameters are particle size, pore size ,internal diameter and pump pressure. The parameters can be changed for different compounds Different steps involved in HPLC Reagents and Chemicals Diosgenin was received by HiMedia. Acetonitrile and Methanol and were of HPLC grade and received from Merck Ltd, New Delhi, India. Instrumentation A Double beam U.V. VIS.

Spectroscopy model of Labindia 3000 plus with 1cm. matched quartz cells was used for determination . The HPLC system (Waters India Pvt Ltd) made up of a pump, a U.V. Visible detector, a Thermo C18 (250 X 4.6 mm, 5µm) column . At specific temperature the chromatographic analysis was performed on a RP-C18 analytical column composed of Acetonitrile: water (92:08 v/v) with a mobile phase and 1 ml per minute was flow rate. In HPLC system for each sample run, a small sample volume of 20 µl was used, being injected .

The chromatogram at a wavelength of 203 nm.was monitored with UV detection . Standard stock solution

Table 3.5 : Selection of Separation Variables

Table 1: Selection of Separation Variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5 µm
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	
Acetonitrile	92
Water	08
Flow rate	1ml/min
Temperature	Ambient Temp.
Sample Size	20 µl
Detection wavelength	203nm
Retention time	4.201± 0.5 min

For the prepration of stock solution of 1000ppm ,10 mg of diosgenin was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the Acetonitrile.

Preparation of working standard solution 1 ml solution was taken from stock solutions of Diosgenin and from this solution diluted up to 10 ml. .1 ml solutions were transferred to 10ml volumetric flasks followed by suitable dilution .Different standard solution of Diosgenin with various concentration prepared. Analysis of Diosgenin in herbal extracts Sample Preparation: 10 mg sample taken in volumetric flask (10 ml)and dilute and mark with Acetonitrile; resultant solution changed into filtered via Whatmann filter paper, sooner or later volume made as much as mark with equal solvent to obtain concentration of 1000 µg/ml.

Identification of marker compound (diosgenin) by HPLC methyl alchohal : acetonitrile (92:08, v/v) was used. Mobile phase equilibrated with a reverse phase C-18 column. Mobile phase flow rate at 1 ml/min was maintained and effluents were monitored at 203nm. Using a 20 µl fixed loop the sample was injected, and the total run time was 10 min.

Table 3.6 : Standard concentration of Diosgenin and generated area under peak

S.N.	Concentration in µg/ml	Area under peak
1	0	0
2	5	423.569
3	10	845.674
4	15	1247.66
5	20	1632.48
6	25	2014.59

The sample solution was chromatographed and a concentration of Diosgenin in Extract sample was found out using regression equation.

Detected percentage of Marker diosgenin in samples Standard Plot for known concentration of Diosgenin Standard with respect to peak values in m volts in particular chromatographic conditions gives the area under peak in order to compare the percentages concentration of marker compound in test

sample extracts.

The Graph is obtained from Excel 2010 linear regression function (figure 4.3). In the test samples extracts of Costus speciosus extracts the percentage of marker compound Diosgenin are depicted in table 3.7

Table 3.7 : The peak values obtained in HPLC chromatogram in the form of mvolts for methanolic extract of naturally grown and tissue cultured comparable with standard to describe the percentage of the marker compound *Diosgenin* in the extracts.

S.N.	Extracts	Concentration (µg/ml)	Peak value (in mvolts)	Percentage Diosgenin
1.	Methanolic extract of Naturally grown plant	1000µg/ml	4.152	0.127
2.	Methanolic extract of tissue cultured plant	1000µg/ml	4.085	0.216

Observation and results

Present study on in vitro culture of Costus speciosus (J.Koen)was investigated to standardize a protocol for the establishment of culture i.e. sterilization of culture , initiation of culture, multiplication of culture, root induction and hardening of micropropagated plants. It was noted that the culture of explants was dependent on plant stage and season of collection. The best result was noted in the month of October to December. **2.To standardize the effect of Growth Regulators for shoot induction of Costus speciosus**

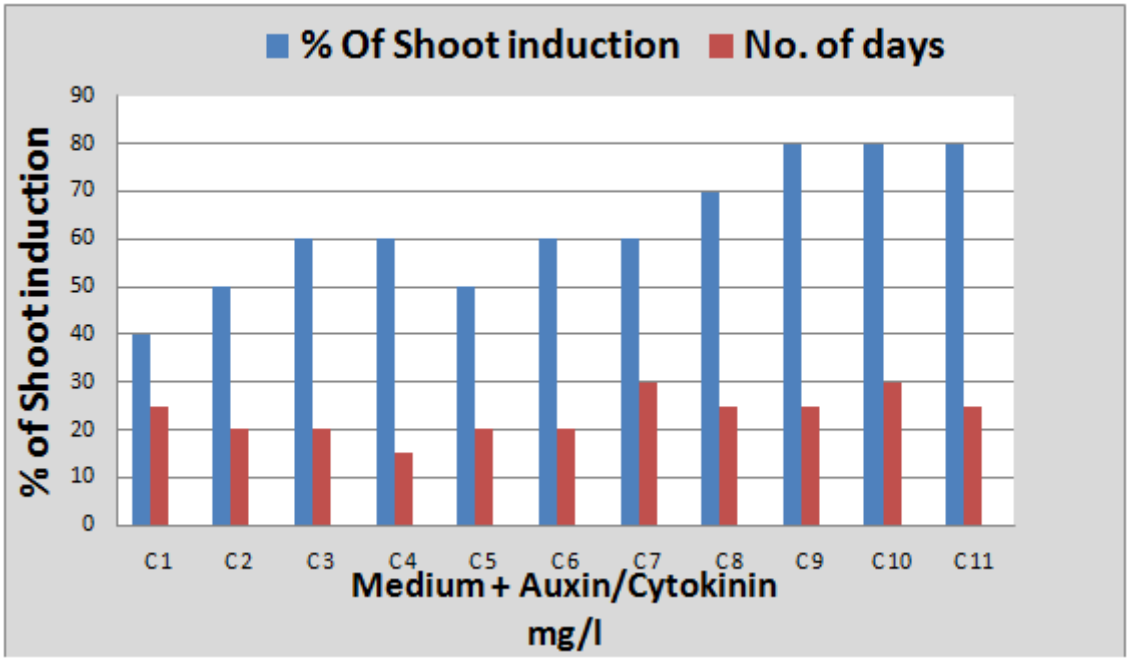
The culture were maintained at 25° C and 16 hour photo period , after 20 days shoot initiation was reported. The initiation of shoots was reported maximum in the 4.0 BAP alone containing. medium After 15 -20 days the maximum number of shoots initiated in the BAP alone containing medium .2 -3 shoots initiated which became 2 – 3 cm long within 20 days. Phytohormone NAA promotes the percentage of shoots initiation. About 1-2 shoots were initiated within 20 days at low concentration of BAP and Auxin.

Table 4.1: Effect of growth regulators on shoot induction of *Costus speciosus*

S.No	Medium MS with GR(mg/lit)	% of shoot induction	No. of days	Number of shoots per explants (Mean ± SE)	Shoot Length in cm (Mean ± SE)
1.	1.0 BAP	40%	25	1.0±2.45	2±0.35
2.	2.0 BAP	50%	20	1.0±0.21	5.0±0.23
3.	3.0 BAP	60%	20	3.0±0.47	2±0.35
4.	4.0 BAP	60%	15	3.0±1.2	2±0.26
5.	1.0 BAP + 0.5NAA	50 %	20	2.7±0.5	2±0.46
6.	2.0 BAP+0.5NAA	60 %	20	2.0±0.4	2.4±0.1
7.	1.0BAP+0.5NAA+1.0KN	60%	30	3±1.80	3±0.38
8.	3BAP+0.5NAA+1.0 KN	70%	25	3.0±1.6	2±0.32

9.	4.0BAP+0.5NAA+1.0 KN	80%	25	4±1.14	3.0±0.36
10.	4.0BAP+0.5NAA+10 AS	80%	30	3±1.44	4.0±0.30
11.	4.0BAP+0.5NAA+ 20AS	80%	25	2±1.05	4.5±0.25

Graph 1 :Effect of MS media and growth regulators on initiation of *Costus speciosus*



Shoots produced when High concentration of Cytokinin and Auxin .

The initiated shoots were transferred in the fresh medium in which BAP alone and with combination of Auxin. The higher multiplication of shoots was reported in 5.0 BAP with combination of 0.5 NAA . Both concentration and combination of growth hormones show multiplication within two weeks of incubation. Addition of AS 20 gm enhance the shoot multiplication .

Multiplication of shoot and elongation growth of shoots was comparatively higher when the medium supplemented with BAP and NAA. After 25-30 days of culture observed shoot length increased faster in the medium about 4-5 shoots of 2 to 3 cm length has been achieved . The multiplication pattern which was observe in Costus speciosus was that the shoots multiplied at the lower end of the explant.

Table 4.2 : Effect of growth regulators on Shoot multiplication of *Costus speciosus*

S.No.	MS Auxin/Cytokinin mg/l	% of Shoot Multiplication	No. of days	Mean no. of shoots	Shoot length in cm. ±SE
C.M.1	3.0 BAP	55	20	4.0 ± 0.5	2.0±0.3
C.M.2	4.0 BAP	60	25	5.0 ± 0.08	2.0±0.22
C.M.3	5.0 BAP	50	15	5.4 ± 0.9	2.0±0.30
C.M.4	6.0 BAP	50	20	5.0 ± 0.1	2.0±0.24
C.M.5	4.0BAP + 0.5NAA	70	25	5.0 ± 0.7	3.0±0.6
C.M.6	5.0BAP+0.5NAA	60	30	4.0 ± 0.5	2.0±0.1

C.M.7	5.0 BAP+0.5NAA+1.0 KN	70	30	4.0 ± 0.5	4.0±0.38
C.M.8	6.0BAP+0.5NAA+1.0KN	70	25	4.0 ± 0.9	4.0±0.32
C.M.9	5.0BAP+0.5NAA+20AS	80	30	6.0± 0.9	4.0±0.32
C.M.10	6.0BAP+0.5NAA+20 AS	70	25	5.0 ± 0.1	4.0±0.30

Graph 2: Effect of growth hormone on Shoot multiplication

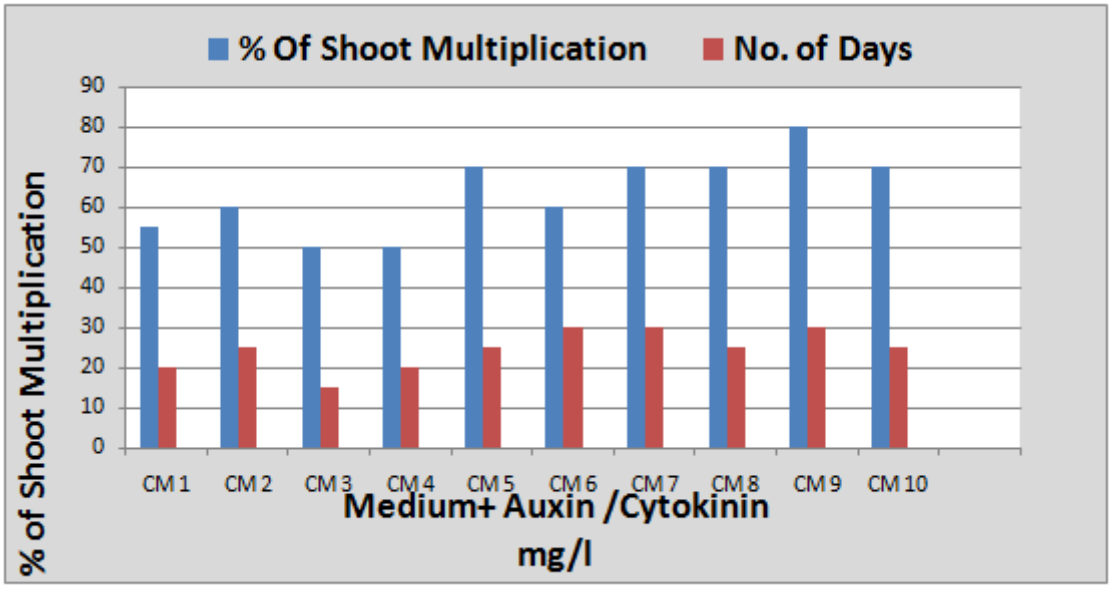
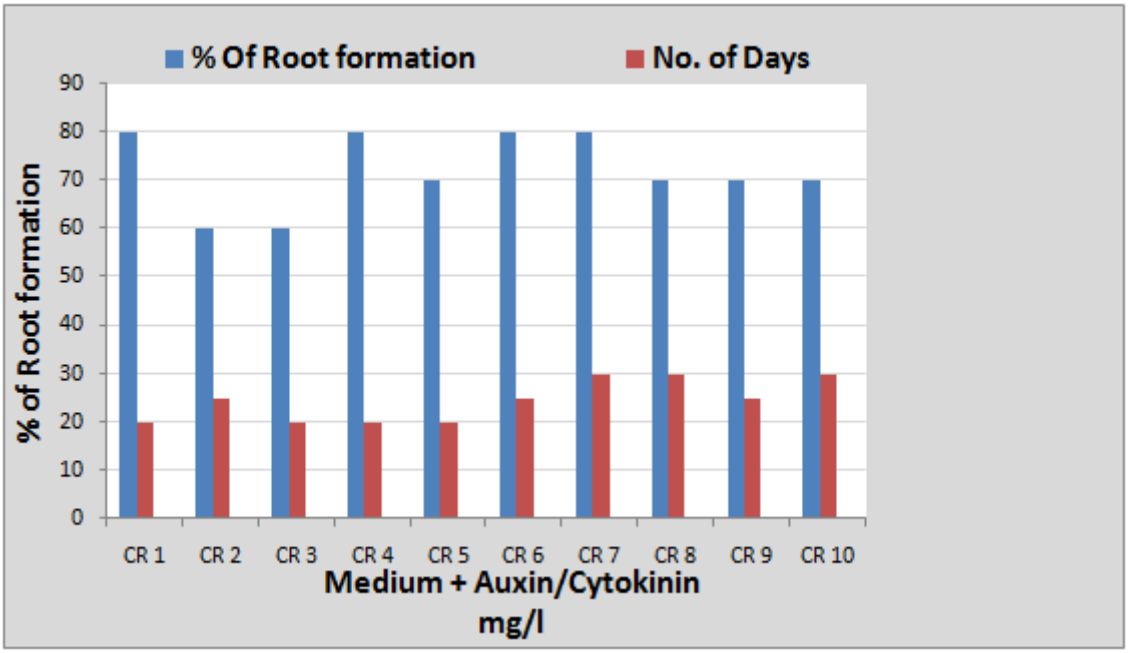


Table 4.3 : Effect of growth regulators on root induction of *Costus speciosus*

S.NO.	Media MS+ GR mg/l	% of root formation	No. of days	Mean Root length in cm± SE
CR 1	MS +10 AC	80%	20	4.0± 0.4
CR2	MS +20 AC	60%	25	3.0± 0.0
CR 3	MS +0.5 IBA	60%	20	5.0± 0.8
CR 4	MS +1.0 IBA	80%	20	4.0 ± 0.6
CR 5	MS+2.0 IBA	70%	20	5.0± 0.6
CR 6	MS+0.5 NAA	80%	25	5.0 ± 0.2
CR 7	MS+1.0 NAA	80%	30	5.0 ± 0.7
CR 8	MS+2.0	70%	30	5.0 ± 0.1
CR 9	MS +0.5 IAA.	70%	25	4.0 ± 0.2
CR 10	MS +1.0 IAA.	70%	30	4.0± 0.2

Graph :3 Effect of growth hormones on root formation of *Costus speciosus*



Reported the root induction when we added 2.0 mg/l NAA and 0.5mg/l IAA in MS media.After few weeks 2-3 cm elongated shoots in length were cut and cultured on MS medium with different combinations of Sugar and agar with MS basal medium. After 25-30 days of inoculation Initiation of rooting took place.

Single and multiple roots Were formed from rhizome and the length of the roots were 1-2 cm within 25-30 days. 0.5 IBA ,0.5 NAA,0.5 IAA different harmone combination taken for study the rooting response .When we added activated charcoal observed highest root induction . Transplantation In vitro grown rooted shoots transfer to soil is very important process. In vitro rooting is very essential for transplantation.

Hardening of plants is very careful and stepwise process. Rooted plantlets are safely pick up from the vessels, adhering agar is removed by careful washing with running tap water and plantlets were transferred in a soil mixture 1:2:1 (soil, sand and vermicompost). After planting, plants are thoroughly watered and maintained humidity.

After 20-25 days plantlets are then transferred to field.

Phytochemical analysis

Phytochemical analysis conducted on the Costus speciosus rhizome extracts. In the Costus speciosus the results represent the presence of important constituents The rhizome extracts have represent the presence of alkaloid, flavonoids, Tannins, Terpenoid and Saponins.

Table 4.5 : Preliminary chemical analysis in *Costus speciosus* extracts

S.NO.	Test conducted	Natural Plant Extract	Tissue cultured Extract
1.	Alkaloid Test	+Ve	+Ve
2.	Flavonoid Test	+Ve	+Ve
3.	Tannins Test	+Ve	+Ve
4.	Glycoside Test	-Ve	-Ve
5.	Terpenoid Test	+Ve	+Ve
6.	Saponins Test	+Ve	+Ve
7.	Anthraquinone Test	-Ve	-Ve

Statement:

The presence of chemical groups tested in extracts were alkaloids, flavonoids, tannins, glycosides, terpenoids, saponins and anthraquinones.

It was observed that out of the 7 chemical groups tested in methanol rhizome extract of Costus speciosus of different origin (natural and plant tissue culture)was observed to contain 5 out of 7 chemical groups. (see. Table 4.5).

- Both natural and tissue cultured plant samples were observed and confirmed the presence of Alkaloid, flavonoids, tannins, saponins and terpenoids in their methanolic extracts, while rest other tested chemical groups are not reported at the time of testing as conducted preliminary chemical analysis.

Observation and results

TLC profiling of standard markers on plate in chromatographic condition / Distance travelled by standard marker saponin, diosgenin and alkaloid colchicin on 2 different TLC plats spotted with 2 different extracts of Costus speciosus. speciosus generating Rf values observable in short & long range UV wavelength under current chromatographic conditions

Table 4.6. : Observation and results TLC profiling of standard markers on plate in chromatographic condition

S.N.	Sample Used	No. of band/spot (At Short wavelength)	No. of band/spot (At Long wavelength)
1	*Diosgenin(std.)*	1	1
2	*Colchicin(std.)*	1	1

Calculation of R_f Value:

$$R_f \text{ (Retention factor)} = \frac{\text{Distance travelled by Spot}}{\text{Distance travelled by Solvent}}$$

Table :4.7 R_f Values of Diosgenin and Colchicine

Distance travelled by standard marker saponin, diosgenin and alkaloid colchicin on 2 different TLC plats spotted with 2 different extracts of C. speciosus generating Rf values observable in short & long range UV wavelength under current chromatographic conditions

S.N.	Plate Tag	Travel distance		Mobile phase distance	R_f Value	
		Diosgenin	Colchicin		Diosgenin	Colchicin
1	Plate 1 & Plate 2	6.3 cm	0.9 cm	7.0 cm	0.9	0.12

Table :4.8 TLC chromatogram details of methanolic rhizome extract of Naturally and tissue cultured *C. speciosus* methanolic extract in chromatography conditions compared to Diosgenin in both short & Long UV wavelengths

Sample extract	*Extract 1 and Extract 2*
Mobile Phase	Toluene: Ethyl acetate: Acetic acid: Formic acid (ratio= 4:3:1:1)
Plate Tag	Plate 1
Spot description from left to right on plate	Diosgenin, extract 1 and extract 2 respectively
Distance travelled by mobile phase	7.0 cm
Number of spots at short & Long wavelength	6 & 7 Respectively
Visibility	Sharp
Distances of spots from start point of each spot (from bottom to top)	1.0 cm, 3.2 cm, 4.2 cm, 5.5 cm, 6.0 cm and 6.3 cm
Any Match with Diosgenin Standard	Yes
R_f Values of each spot (from bottom to top)	0.14, 0.45, 0.5, 0.6, 0.78, 0.85 and 0.9 respectively

Table 4.9: TLC chromatogram details of methanolic rhizome extract of Naturally and Tissue cultured *C. speciosus* methanolic extract in chromatography condition compared to Colchicin in both short & Long UV wavelength

Sample extract	*Extract 1 and Extract 2*
Mobile Phase	Toluene: Ethyl acetate: Methanol: Ammonia (ratio= 30:20:20:0.1)
Plate Tag	Plate 2
Spot description from left to right on plate	Colchicin, extract 1 and extract 2 respectively
Distance travelled by mobile phase	5 cm
Number of spots at short & long wavelength	5 & 6 Respectively
Visibility	Sharp
Distances of spots from start point of each spot (from bottom to top)	0.9 cm, 1.5 cm, 2.9 cm 3.8 cm, 5.0 cm and 5.6 cm
Any Match with Colchicin Standard	Yes
R_f Values of each spot (from bottom to top)	0.12, 0.21, 0.41, 0.54 0.71 and 0.8 respectively

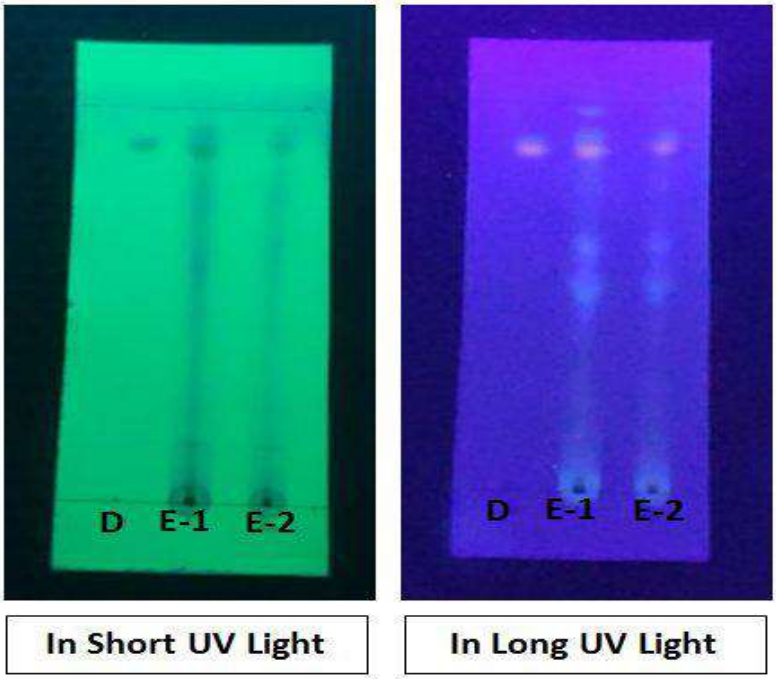


Figure 4.1: Visualization of TLC plates in **Short & Long Range UV light** of plate 1 loaded with methanolic extracts of both naturally grown and tissue culture raised Costus speciosus rhizomes compared with standard diosgenin

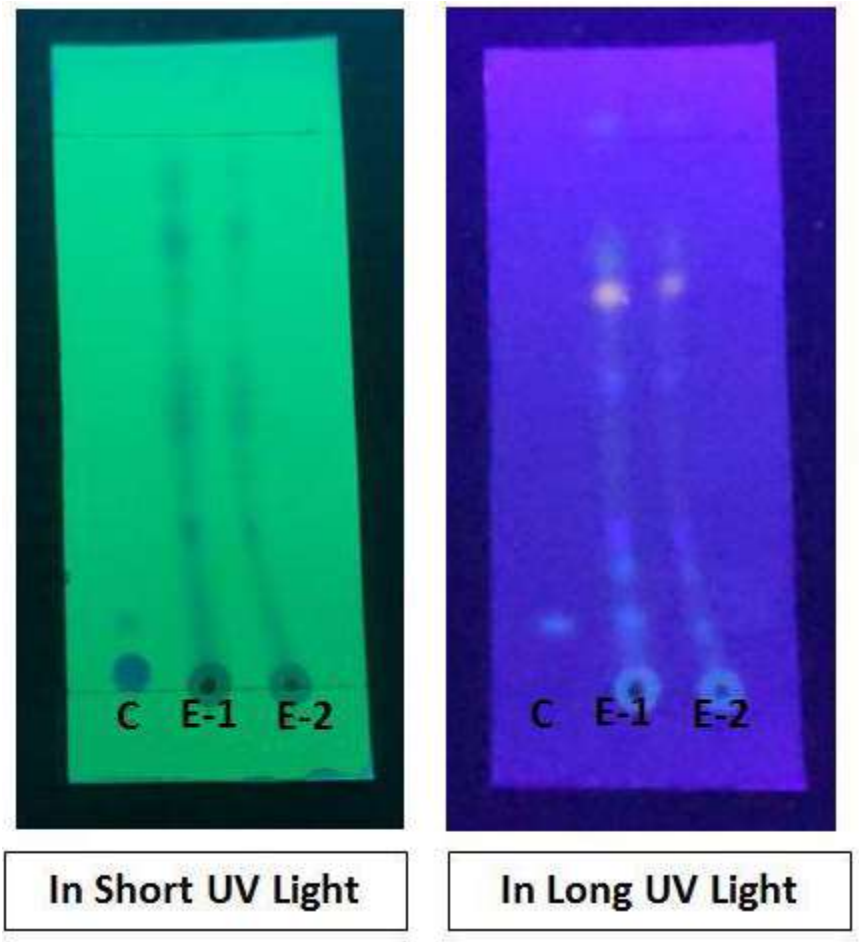


Figure 4.2: Visualization of TLC plates in **Short & Long Range UV light** of plate 1 loaded with methanolic extracts of both naturally grown and tissue culture raised *Costus speciosus* rhizomes compared with standard Colchicin .

Statements:

The chromatographic conditions used in TLC analysis of methanolic rhizome extract of of Costus speciosus of both naturally grown and tissue culture grown plants shows similar spot separation pattern when visualized under short UV range and long UV range for both diosgenin and colchicine marker detection on two separate plates whose chromatographic conditions are mentioned in table 4.9 and table 4.10 of this report.

*Under

chromatographic condition as mentioned in table 4.9 for detection of Diosgenin in extract 1 and extract 2 which are from naturally grow and tissue culture grown plant origin respectively suggest that there must be different chemical constituent confirmed in the extracts with considerably clearly visible spots from bottom to top of the TLC plate.

*One of the spot from both the extracts matches with the standard diosgenin spot at the distance of 6.3 cm from the bottom confirmed diosgenin like compound in both the extracts.

* Again referring to table 4.10 under particular chromatographic conditions the spots of both extract 1 and extract 2 gets separated into total number of 6 different spots which confirmed the presence of 6 different type of chemical constituents which gets separated under certain chromatographic conditions

used in present experiment on TLC plate 2.

*Out of the 6 separated spots on comparison with standard colchicine spot which travelled up to the level of 0.9 cm from the starting point at the bottom was reported to be matching with one of the spots developed at the same range due the constituent separation in both extract 1 and extract 2 on plat 2 indicates the presence of colchicines line alkaloids in the methanolic extracts from plant of both natural and tissue culture origin.

Quantitative HPLC Study for Diosgenin Marker Compound High Performance Liquid Chromatography: (HPLC) HPLC is also known as High- Pressure Liquid Chromatography. HPLC is the combination of a suitable stationary phase and mobile phase. The mobile phase for HPLC is the liquid phase which is continually flowing through stationary phase . On the basis of their interaction with solid particles , tightly packed column and the solvent of the mobile phase separates compounds . Quantitative and qualitative analysis by HPLC . HPLC provides both in a single operation .

Table 4.10:Selection of Separation Variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5 µm
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	
Acetonitrile	92
Water	08
Flow rate	1ml/min
Temperature	Ambient Temp.
Sample Size	20 µl
Detection wavelength	203nm
Retention time	4.201± 0.5 min

Observed Results of High Performance Liquid Chromatography Identification of marker compound (diosgenin) by HPLC.

Mobile phase methanol: acetonitrile (92:08, v/v) equilibrated with A reverse phase C-18 column was used. The sample solution was chromatographed and a concentration of Diosgenin in Extract sample was found out using regression equation.

Table 4.11: Standard concentration of Diosgenin and generated area under peak

S.N.	Concentration in µg/ml	Area under peak
1	0	0
2	5	423.569
3	10	845.674
4	15	1247.66
5	20	1632.48
6	25	2014.59

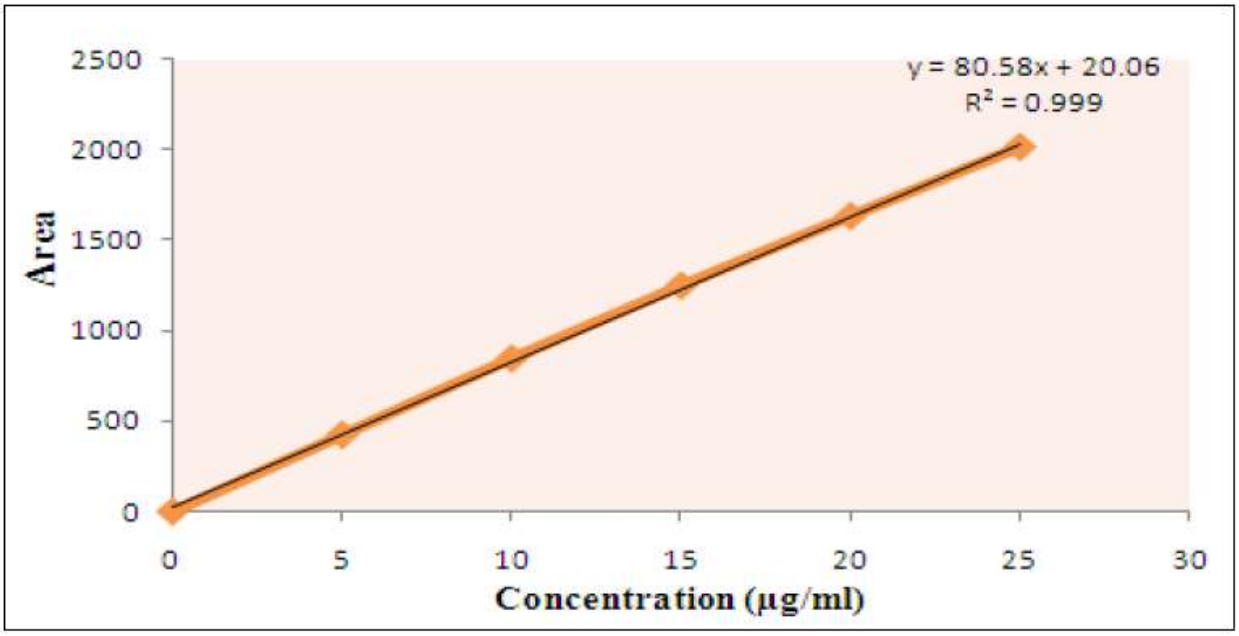


Figure 4.3: Standard plot of marker compound Diosgenin developed area under peak and concentration

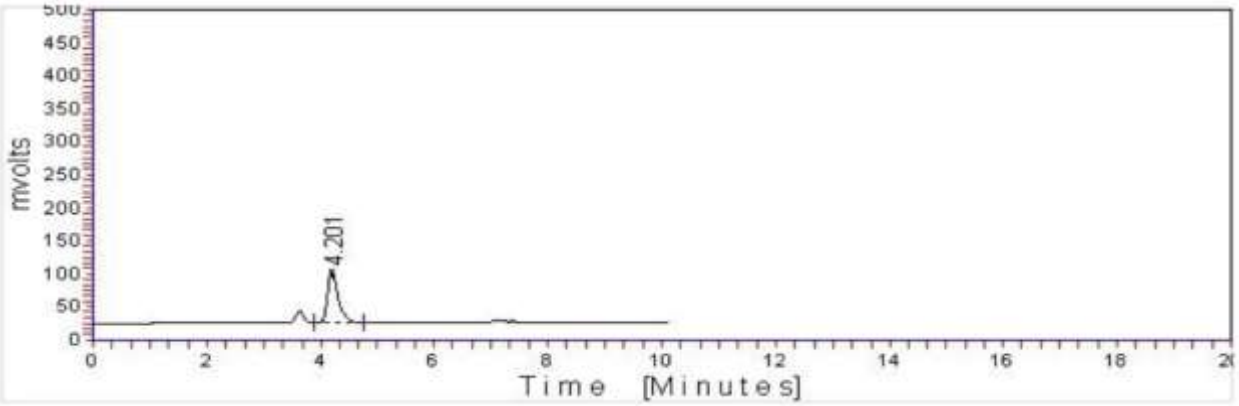


Figure 4.4: Chromatogram of standard Diosgenin marker

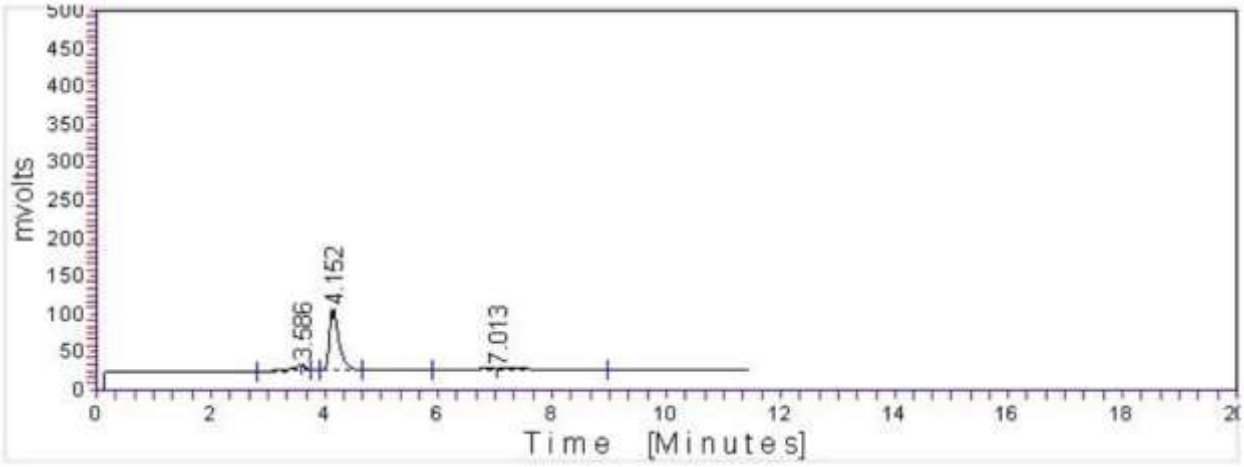


Figure 4.5: Chromatogram of methanolic extract of naturally grown Costus speciosus for detection of Diosgenin marker

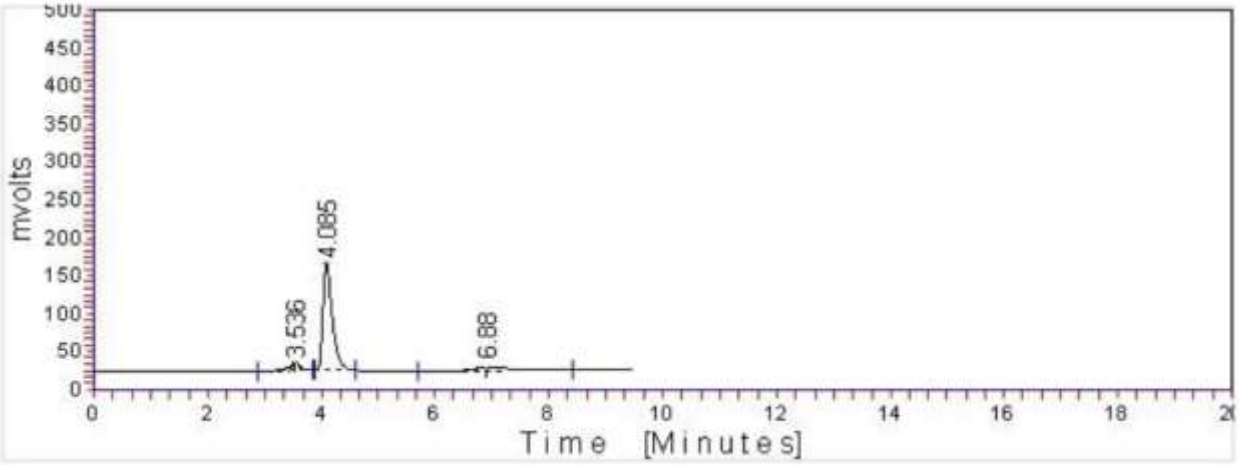


Figure 4.6: Chromatogram of methanolic extract of tissue cultured *costus speciosus* for detection of Diosgenin marker

Detected percentage of Marker diosgenin in samples

Standard Plot for known concentration of Diosgenin Standard with respect to peak values mvolts in particular chromatographic conditions gives the area under peak in order to compare the percentages concentration of marker compound in test sample extracts.

Table 4.12: The peak values obtained in HPLC chromatogram in the form of mvolts for methanolic extract of naturally grown and tissue cultured comparable with standard to describe the percentage of the marker compound *Diosgenin* in the extracts.

S.N.	Extracts	Concentration (µg/ml)	Peak Values (in mvolts)	Percentage *Diosgenin*
1	methanolic extract of naturally grown	1000 µg/ml.	4.152	0.127
2	methanolic extract of tissue cultured	1000 µg/ml.	4.085	0.216

The Graph is obtained from Excel 2010 linear regression function (4.3). In the test samples extracts of rhizome the percentage of marker compound Diosgenin are represented in table of this section C of this report.

Statements:

It is observed that, the methanolic extracts rhizome of plant Costus speciosus from both the sample i.e., plant of natural origin and tissue cultured plant samples responded for detection of diosgenin like saponins in samples during HPLC analysis.

- In HPLC techniques the detection of the presence of saponin in terms of diosgenin marker compound it reported to be positive, though its percentage concentration is quite low in both the samples analysed.
- Inspite of the low concentration, the diosgenin percentage is reported to be higher in tissue culture samples than in natural plant samples.
- The percentage of Diosgenin in methanolic extract of naturally grown was reported to be 0.127% while the percentage of Diosgenin in methanolic extract of tissue cultured was reported to be 0.216 % during HPLC analysis.

Summary

Some plants are rich source of secondary metabolites which imparts them medicinal properties. Such plants have a valuable place in traditional herbal medicine.

Those plants which posses secondary metabolites known as medicinal plants and these are very important commercially. Costus speciosus plants has medicinal and pharmacological properties . Costus speciosus belongs to family Zingiberaceae (Costaceae) , posess medicinal properties and grown as ornamentally .

Costus speciosus plant commonly called as insulin plant (antidiabetic plant) .This plant has many innumerable medicinal properties so over exploited. Presently the status of Costus speciosus be noted endangered. In vitro micro-propagation are very useful for conservation of endangered species. Costus speciosus rhizomes are edible . Famous vegetable in South –East Asia. Rhizome, shoots and fruits used as vegetables.

Diosgenin is an important secondary metabolite present in Costus speciosus plant. Many other secondary metabolites also reported from stem, rhizome and leaves .The rhizome extract is used for leprosy, asthma, bronchitis, anemia and other skin diseases. For treatment of stomach disorder and scabies leaves are used. Stems paste applied for blisters. Rhizome extract is used for treating snake bites.

Costus speciosus posses antiseptic, diuretic, hypoglycemic and digestive properties. The rhizome is very effectivein the treatment of jaundice, abdominal pain, gall bladder pain and liver problem. Costus speciosus exhibited antifungal, antipyretic, anti-helminthes, anti-fertility and anti-inflammatory activities.

Costus speciosus vegetatively propagated through pieces of rhizomes and stems. Multiplication rate is very slow, poor seed viability and low germination percentage . These are traditional technique which cannot fulfill the needs Recently protection of Costus speciosus by micropropagation is a initiative step to conserve the endangered medicinal plants. Using tissue culture techniques of micro-propagation, it is possible to produce numerous plants under aseptic conditions.

In India several important and necessary steps have to be taken up for its propagation. Tissue culture represent best results in multiplication of endangered plants . Rhizome and stem segments were selected

as explants for in vitro micro-propagation . Explants Surface sterilization with 0.1% $HgCl_2$ is the best treatment .It was observed that rhizome explants is suitable for Costus speciosus establishment. The explants cultured on media contained with growth hormones .The development of plant depend upon selection of culture media. Appropriate nutrient medium is a starting point of organogenesis .Organogenesis is controlled mostly by a balance between cytokinin and auxin.

For micro-propagation explant rhizome has been used as the starting point BAP alone or used in combination with NAA and AS .High Shoot initiation percentage observed when 4.0 BAP , 0.5 NAA and 10 AS were used .After 1 week initiated shoots were taken and shifted to different percentage multiplication medium. High concentration of phytoharmones 5.0 BAP,0.5

NAA and 20 AS has been used. Percentage of shoot response, number of various shoot and shoot length measurement was taken . The multiplied shoots inoculated in the activated charcoal containing medium shows 80-90% rooting.Well developed long roots were increased with the age of culture. Each plantlet hardened .High humidity maintained. After few weeks micropropagated raised plant with rhizome developed.

The presence of chemical groups tested in extracts were alkaloids, flavonoids, tannins, glycosides, terpenoids, saponins and anthraquinones. It was reported that out of the 7 chemical groups tested in extract of Costus speciosus rhizome in methanol of different origin (natural and plant tissue culture) was observed to contain 5 out of 7 chemical groups.

Both natural and tissue cultured plant samples were reported the presence of flavanoids, tennins, terpenoids and saponins in their methanolic extract. Diosgenin is a main bioactive component present in Costus speciosus. In TLC analysis rhizome of Costus speciosus of both naturally grown and tissue culture grown plants in methanolic extract shows similar spot separation pattern when visalized under short UV range and long UV range for both diosgenin and colchicines marker detection on two separate plates whose chromatographic analysis represent the presence of alkaloids and diosgenin in the methanolic extracts from plant of both natural and tissue culture origin.

HPLC screening showed the presence of diosgenin and alkaloid.HPLC study is taken up to estimate the amount of diosgenin present in rhizome of Costus speciosus. The diosgenin percentage is reported to be higher in tissue culture samples than in natural plant samples. The percentage of Diosgenin in methanolic extract of naturally grown was reported to be 0.127% while the percentage of Diosgenin in methanolic extract of tissue cultured was reported to be 0.216% during HPLC analysis.

The present work developed the protocol to micropropagate the endangered species Costus speciosus from rhizome and comparative phytochemical study between natural plant and tissue raised plant . A major bioactive component diosgenin is present in Costus speciosus. The present study using HPLC analysis estimate the amount of diosgenin and alkaloid present in the rhizome of Costus speciosus.

Conclusions

Medicinal plants are being used from past times to treat diseases, to maintain health and maintain the healthy state of mind and body. Herbal medicines are very effective . Pharmaceutical companies used Costus speciosus as a medicinal plants. In recent investigation different secondary metabolites reported from Costus speciosus especially diosgenin.

Due to over exploitation its necessary to conserve the Costus speciosus species by in vitro culture .In tissue culture technique small explant can develop multiple clones. Costus speciosus (J.Koen.) belong to family Costaceae. Plant are very useful for treatment of different diseases .We can regenerate new

plants from rhizome. Rhizome extract used as a tonic, purgative and expectorant.

Many properties such as antioxidant, antibacterial, antibacterial, antihyperglycemic, antipyretic, antidiuretic, antistress and anti-inflammatory has been reported in Costus speciosus. High demand of Costus speciosus in the market . Alternative propagation method may conserve the Costus speciosus plant. In the present research work we have attempted to develop suitable micropropagation protocol. In the recent work following conclusions has been reached.

1. Following sterilization protocol of Costus speciosus explants was found satisfactory. Under plain water washing of rhizome explants for 30 min. (2 cycle) Rhizome part were dipped in aqueous solution of savlon for 10 min. In solution containing Bavistin rhizome part were soaked for 10 min. After 10 min. it is washed with double distilled water for 4 minutes for 2 cycles. Now it is treated with mercuric chloride(0.01%)aqueous solution for 3 min. 1.Explants thoroughly washed for two times with double distilled water and inoculated in the culture medium.
2. The best medium for initiation MS medium with growth hormones. The maximum 80% shoot initiation were observed in BAP, NAA and AS. MS media with NAA and BAP shows best multiplication. MS medium with growth hormone represent the best root initiation . Transplantation was done by using soil , mixture soil and sand.
3. Mostly Secondary metabolites are accumulated by plant cells in smaller quantities than primary metabolites. Costus speciosus (J.Koen) medicinal plant rich in secondary plant products. It is a main source of several natural drugs.Percentage of secondary metabolites which are present in different parts of plants greatly affected by different factors .
4. In Costus speciosus various secondary metabolites alkaloid, flavonoids, tannins, saponins and terpenoids observed by phyto-chemical analysis. Diosgenin secondary metabolite is a major bioactive constituent found in this plant & its concentration is maximum as compared to others.
5. **Thin layer chromatography (TLC)** – The identity of compound and the purity of compound can be determined by TLC. it is relatively rapid and microgram quantities can be analyzed by TLC. In present study the RF values of Diosgenin and colchicines were measured. TLC analysis of both naturally grown and tissue cultured raised plant rhizome extract showed same pattern of spot separation on TLC plate. RF values observed under short UV length and long UV length. RF value of Diosgenin is 0.9 and RF value of colchicine is 0.12. 5.
6. **High performance liquid chromatography (HPLC)-** HPLC is an analytical technique and it is high sensitive technique. HPLC technique was used to quantify Diosgenin .According to recent research work percent of Diosgenin is higher in tissue culture sample than in natural plant samples. The percentage of Diosgenin in methanolic extract of naturally grown was reported to be 0.127% while the percentage of Diosgonin in methanolic extract of tissue cultured was reported to be 0.216% during HPLC analysis.

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